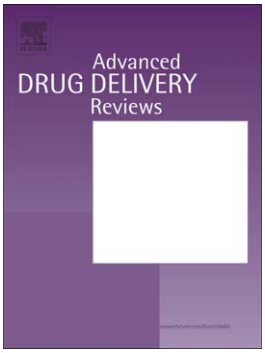

Designer Bacteria as Intratumoural Enzyme Biofactories

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Title: Designer Bacteria as Intratumoural Enzyme Biofactories

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Tumor; DEPT; Prodrug; Cancer; Chemotherapy; Drug design; Synthetic biology; Targeted; Translational research; R&D

Abstract

Bacterial-directed enzyme prodrug therapy (BDEPT) is an emerging form of treatment for cancer. It is a biphasic variant of gene therapy in which a bacterium, armed with an enzyme that can convert an inert prodrug into a cytotoxic compound, induces tumour cell death following tumour-specific prodrug activation. BDEPT combines the innate ability of bacteria to selectively proliferate in tumours, with the capacity of prodrugs to undergo contained, compartmentalised conversion into active metabolites *in vivo*. Although BDEPT has undergone clinical testing, it has received limited clinical exposure, and has yet to achieve regulatory approval. In this article, we review BDEPT from the system designer's perspective, and provide detailed commentary on how the designer should strategize its development *de novo*. We report on contemporary advancements in this field which aim to enhance BDEPT in terms of safety and efficacy. Finally, we discuss clinical and regulatory barriers facing BDEPT, and propose promising approaches through which these hurdles may best be tackled.

Abbreviations

5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; 6-MeP, 6-methylpruine; 6-MeP-dR, 6-methylpurine-2-deoxyriboside; 9-AC, 9-aminocamptothecin; ACV, acyclovir; ADEPT, antibody-directed enzyme prodrug therapy; API, active pharmaceutical ingredient; BCG, bacillus Calmette-Guerin; BDEPT, bacterial-directed enzyme prodrug therapy; β-G, β-glucuronidase; CD, cytosine deaminase; CNOB, 6-chloro-9-nitro-5-oxo-5H-benzo(a)phenoxazine; CPG2, carboxypeptidase G2; DNA, deoxyribonucleic acid; FIAU, 2'-fluoro-1-beta-D-arabino-furanosyl-5-iodo-uracil; GCV, ganciclovir; GDEPT, gene-directed enzyme prodrug therapy; HSV-1, herpes simplex virus type 1; LPS, lipopolysaccharide; MCHB, 9-amino-6-chloro-5H-benzo(a)phenoxazine-5-one; MI, molecular imaging; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NTR, nitroreductase; OI, optical imaging; PET, positron emission tomography; PNP, purine nucleoside phosphorylase; R&D, research and development; RNA, ribonucleic acid; TK, thymidine kinase; UPRT, uracil phosphoribosyl transferase; VDEPT, viral-directed enzyme prodrug therapy

Introduction

Off-target toxicity is a key limitation in cancer chemotherapy, where the concentration of drug usually required for efficacy is too toxic to the patient, inducing unacceptable side effects and preventing tumour clearance. To overcome this, several substitute strategies have been developed over the years which aim to localise treatment to the site of the tumour, thus sparing healthy tissue. One such strategy involves gene therapy, whereby drug delivery vehicles are engineered to carry nucleic acids to tumour cells, encouraging the production of anticancer biomolecules. Early gene therapies relied heavily on viruses to serve as gene carriers [1, 2], but the scope of what can be regarded as a carrier has since expanded beyond viral vehicles to include bacteria. The use of bacteria in treating cancer, however, predates discussion of gene therapy by decades. It was initially pioneered over a century ago by William Coley, after he observed that some cancers of hospitalised patients regressed following accidental *Streptococcus pyogenes* infections, and by Friedrich Fehleisen, who had begun treating oncology patients with the same microbe during the same period [3, 4]. These accounts prompted further research on bacteria in the treatment of cancer, although, as this method was not at the time curative, it was gradually abandoned.

In the mid-20th century, it became apparent that bacteria can preferentially accumulate within tumour tissue. For example, the systemic injection of *Clostridium tetani* spores into mice led to the exclusive localisation of their vegetative form in murine tumours [5]. It has since emerged that some bacteria possess inherent anticancer attributes, without the requirement for 'arming' them with heterologous genes. Intracellular replication of invasive bacteria (e.g. salmonellae) within cancer cells can induce apoptosis, while certain species-specific toxins have been identified, such as with propionibacteria, which can induce apoptosis in colorectal carcinoma cells with fatty acids that are naturally produced by this genus [6]. Similarly, unmodified *Lactococcus lactis* is cytotoxic to cancer cells and impedes proliferation in several cancer cell lines [7]. With the birth of molecular biology in the late 20th century, interest in bacteria as anticancer agents has reignited in earnest. The field is continually evolving, and different preclinical approaches exist which investigate bacteria as tumour-selective entities [8]. Rather than simply viewing bacteria as unmodifiable agents for treating cancer, as they were recognised during Coley's and Fehleisen's time, we have since re-considered their flexibility and have postulated their usability in conjunction with other, more modern types of cancer treatment.

One such treatment type is gene-directed enzyme prodrug therapy (GDEPT), which uses gene therapy techniques to enzymatically convert inactive prodrugs to their active drug counterparts [9]. GDEPT is a growing discipline that was initially conceived to invoke differential expression of enzymes in tumour tissue compared with healthy tissue for medicinal purposes. It essentially encapsulates the tumour-specific expression of a gene, coding for a prodrug-converting enzyme, which logistically leads to the tumour-specific activation of an administered prodrug. Different manifestations of GDEPT exist, characterised by gene delivery that is assisted by carriers predisposed to tumour entry, such as viral-directed enzyme prodrug therapy (VDEPT) [10]. Gene expression can be controlled by ensuring that transcription occurs via a promoter operating only within tumour cells [11]. This promoter can be either a constituent of the carrier's genome or part of the tumour cell's default machinery.

The union of bacteria and GDEPT yields bacterial-directed enzyme prodrug therapy (BDEPT), also referred to as bacterial prodrug therapy, which constitutes a two-step procedure for treating cancer using bacteria as *in situ* sources of prodrug-converting enzymes [12]. In the first phase of treatment, bacteria, that have been armed with a gene coding for an enzyme that can convert an inert prodrug into a cytotoxic active pharmaceutical ingredient (API), are administered to the patient. The bacteria travel to the tumour site, preferentially colonise it, and then begin to proliferate. Once intratumoural bacterial numbers are adequate, and enzyme production amplified, the second step is initiated by administering the prodrug. Following administration, the prodrug spreads systemically throughout the body, but is primarily activated in the tumour by the locally-produced enzyme. The API diffuses then throughout the malignancy, relieving healthy tissue of any damage. As the active drug is generated locally inside the tumour, this approach can be thought of as *in situ* chemotherapy (Figure 1).

The administration of a prodrug has enormous benefits over administering a drug pre-formulated as an API. Ideally, prodrugs are synthesised with certain molecular features that conceal an underlying pharmacodynamic mechanism of action [13]. These features often bestow upon the drug improved pharmacokinetic qualities, augmenting its bioavailability *in vivo*. Described as a "chemistry-enabled drug delivery tool", prodrugs are metabolised to their active form, following administration, by either endogenous or exogenous enzymes [14]. As the core therapeutic objective which GDEPT seeks to accomplish revolves around the confinement of tissue damage to tumours, the use of prodrugs activated by endogenous

enzymes is inadvisable. This is because the prodrug is liable to encounter the enzyme en route to the tumour, resulting in extra-tumoural toxicity [15]. Contrastingly, a prodrug which is activated by an exogenous enzyme gives the clinician significant control regarding the calibration of the patient's treatment, allowing them to time the site-specific initiation of therapy, if the enzyme is guided to the tumour appropriately.

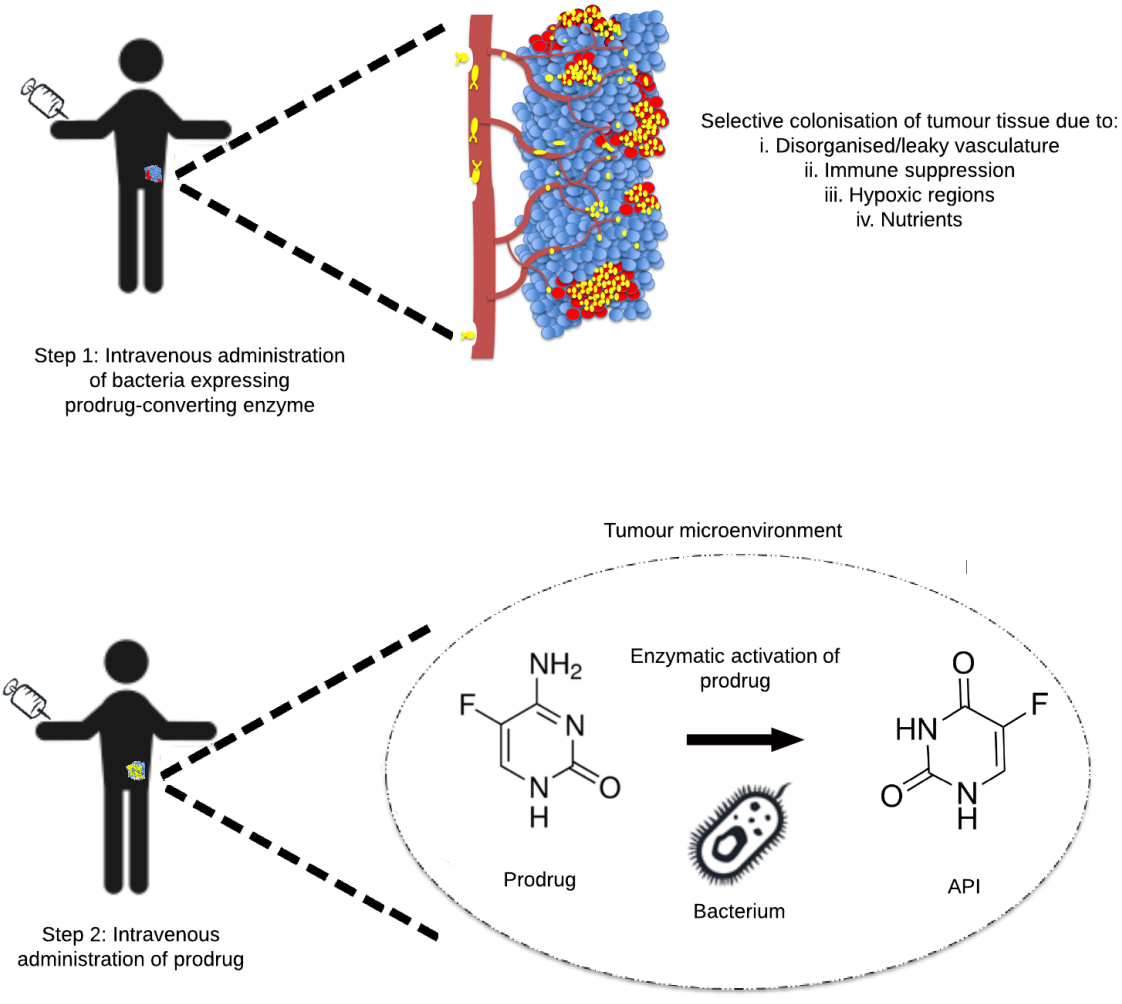

Figure 1. Bacterial-directed enzyme prodrug therapy. *The two-step procedure of BDEPT is illustrated, wherein bacteria expressing a prodrug-converting enzyme are administered to an oncology patient (step 1), followed by systemic administration of a corresponding prodrug when the tumour is adequately colonised (step 2). The in situ bacterial-mediated conversion of 5-fluorocytosine (a prodrug) to 5-fluorouracil (an API) is illustrated in step 2.*

The Bacterial Biofactory: Bacteria as Enzyme Delivery Vehicles

Bacteria have many characteristics which favour them as tumour-targeting agents over other transduction and transfection methodologies. Firstly, they are naturally tumour-selective. A diverse panel of bacteria, including Gram-positive, Gram-negative, aerobic, and anaerobic strains, has illustrated the selective colonisation of various tumour models by all microbes under study [16]. Secondly, bacteria proliferate post-delivery, unlike replication-defective viruses, such as adenoviruses, and non-viral carriers [17-19]. Thirdly, the genomic capacity of bacteria is far greater than that of viruses, making them more amenable to genetic engineering [20]. In fact, a range of therapeutic biomolecules has been used in conjunction with harmless or mildly toxic bacteria, ranging from cytokines to antigens [21-24]. Finally, in the case of emergency, bacteria can be cleared from the patient by antibacterial agents [25] - something which is impossible with other gene therapy carriers.

The nature of tumour-selective colonisation is dependent on a multitude of factors, many of which relate to passive features of malignant tissue. The abnormal, porous vasculature of the tumour facilitates penetration of bacteria, in which they lodge and begin to proliferate, protected naturally from immune surveillance [26]. Immunity serves to quickly clear extra-tumoural bacteria from the bloodstream and non-malignant tissue following administration to humans [25, 27] and animals [28-30], but it also complicates tumour-specific colonisation of the carrier. Threshold administration quantities of microbes to induce colonisation are apparent, and it is likely that once a threshold is exceeded, host innate immune defences are insufficient to neutralise all microbes before they encounter the malignancy [16, 31]. Indeed, while even obligate anaerobic bacteria, such as *Bifidobacterium breve* UCC2003, have been recovered from healthy organs distal to tumours, tumour-to-liver colonisation ratios in the region of 10000:1 have been reported for these microbes [32]. Some bacterial genera have been shown to exclusively colonise tumour areas characterised by hypoxia or anoxia, while others are capable of colonising both necrotic and normoxic tissues [33, 34]. Growth usually occurs in multiple groups of colonies inside the tumour [35, 36]. The proliferation of bacteria *in vivo* is contingent on the availability of certain nutrients such as iron [37]. This dependence can be exploited by host immune defences by curtailing the availability of such nutrients, to counteract the accumulation of bacteria [38]. Not only do tumours protect microbes within it from host defences, but tumour tissues hold a wealth of nutrients, such as amino acids, that provide vital sustenance for bacteria, especially in the case of auxotrophic mutants [30, 39]. There is some evidence suggesting that bacteria are attracted to tumours (or their intratumoural spread is increased) chemotactically by compounds present in quiescent and

necrotic cancer cells [40], propelled there by their own motility [41]. It has also been purported, contrarily, that motility has a negligible effect on bacterial migration to tumours [42]. Colonisation correlates positively with tumour size [43], probably because large, advanced tumours contain marked hypoxia, amenable to more extensive colonisation [44]. However, some tumours as small as 20 mm^3 have proven to be colonisable by bacteria [26].

The unification of bacterial systems and enzyme-mediated prodrug activation is ideal. Commensal bacterial genes typically code for enzymes, and not toxins, and thus are unlikely to be lethal to the host [45]. Furthermore, in the event of bacterial leakage from the tumour into the bloodstream, iatrogenic effects of the API are minimised, as these microbes will be rapidly and systemically eliminated from non-malignant tissue by the immune system. Pathogenic bacteria, such as *Clostridium difficile*, having an innate ability to kill healthy host cells through exotoxin production, can kill tumour cells when in proximity [46]. Despite the tumour-selective growth of bacteria, the non-specific toxicity of pathogenic microbes does not provide a therapeutic index wide enough for safe and effective BDEPT [5]. Modified strains with reduced virulence, or inherently non-pathogenic bacteria, present an opportunity to circumvent this. For example, *Clostridium novyi*, heat-treated to abolish one of its lethal toxins, brought about tumour regression in mice following administration, probably due to the release of oncolytic products from the bacterium [47]. The attenuated strain *C. novyi*-NT has been explored preclinically in combination with other tumour-targeting entities, such as liposomally-encapsulated chemotherapies, for its natural facility to enzymatically release the contents of liposomes [48]. This strain has also been combined with more conventional forms of cancer treatment, including radiation therapy, as a means of specifically targeting hypoxic tumour regions, against which radiation is known to be less effective [49]. Currently, *C. novyi*-NT is being clinically studied in patients with cancers that are refractory to standard treatment (clinicaltrials.gov identifier: NCT01924689) [50].

Constructing a Successful BDEPT: Design Principles

Putting aside everything which makes BDEPT promising, it has yet to achieve a place in the standard anticancer repertory of the clinic, and it is worth examining why this is so. BDEPT is not by any means a “traditional” kind of treatment, and it is unlike any form of anticancer therapy currently in clinical use. Non-targeted chemotherapies and monoclonal antibodies, for instance, are comprised of chemical functional groups and amino acids, and are not reliant

on living delivery vehicles for conveyance to their sites of action. BDEPT designers, having no blueprint of what a truly effective BDEPT really looks like yet, are compelled to feel their way as they search for successful therapies of this kind. It is therefore imperative to have a solid understanding of all design variables and how best to tailor them for optimisation of safety and efficacy of this platform.

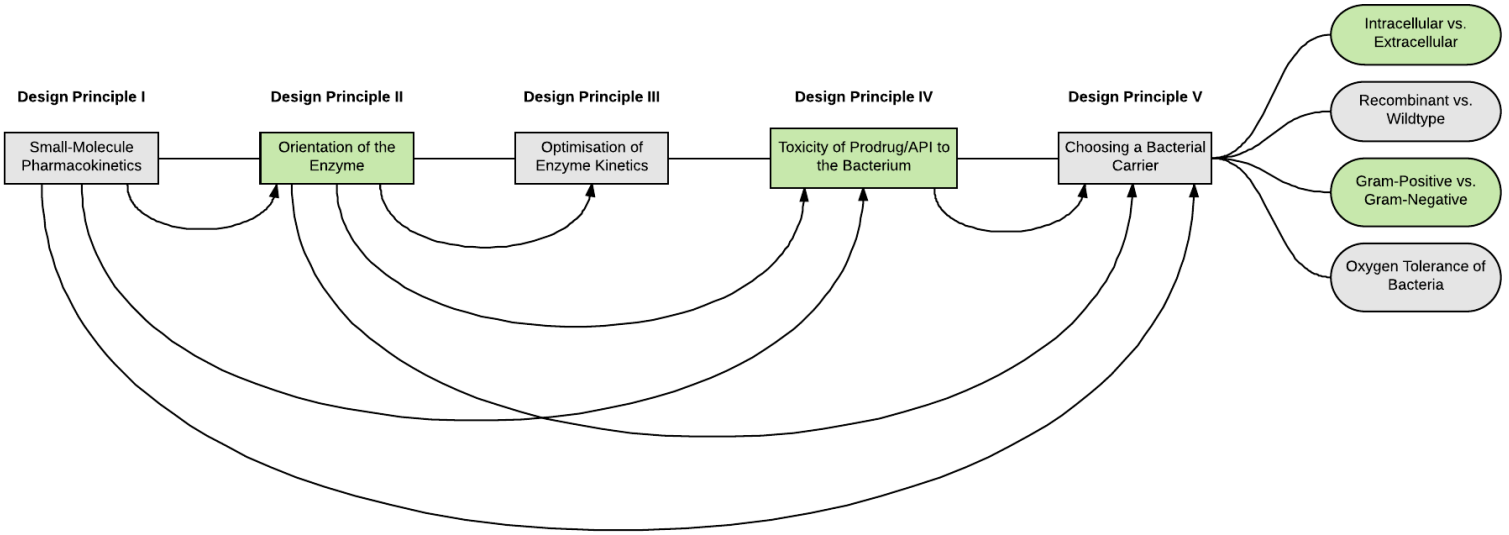

Figure 2. BDEPT Design Process Flow. *An illustration of the order in which the de novo design of BDEPT should be approached. The process is divided into five distinct design principles. Curved arrows pointing from one design principle to another indicate a relationship in which an understanding of the design principle being pointed to is dependent on a familiarity with the design principle from which the arrow comes.*

Design Principle I: Small-Molecule Pharmacokinetics

The most pertinent questions to pose when designing a BDEPT concern the pharmacology of the prodrug and its corresponding API. Fundamental questions about the distribution pharmacokinetics of both drug and prodrug should be considered. It is crucial that these behaviours are adequately defined from the outset, because this will guide many of the ensuing steps of BDEPT design, including the correct alignment of the bacterial enzyme (Design Principle II), management of toxicological issues (Design Principle IV), and the selection of an appropriate bacterial carrier (Design Principle V) (Figure 2). The designer must know how easily their compound can infiltrate a cell by crossing the cell membrane. A prodrug will be converted to an API by the intracellular enzyme of a bacterium only if it can enter the bacterial cytoplasm. Furthermore, an API, which relies on direct engagement with intracellular tumour elements to function, must be present within a tumour cell for its mechanism of action to take hold. For example, 5-Fluorouracil (5-FU), an API commonly employed in BDEPT, acts as an inhibitor of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis only when it is processed intracellularly by mammalian enzymes [51].

Pharmacokinetic properties can vary considerably between drug and prodrug. They can change so markedly upon activation that a prodrug which is capable of crossing cell membranes with ease can become impeded from doing so once enzymatically metabolised, thereby negating or reducing its capacity as a chemotherapeutic agent [14]. If an API is generated external to a tumour cell, and its cell membrane is impenetrable to the API, the drug will be unable to initiate its anticancer modality. In these scenarios, prodrug conversion must occur within tumour cells for therapy to be effective. Conversely, an API which crosses cell membranes easily can undergo activation extracellularly while retaining therapeutic efficacy.

Fortunately, the properties of small-molecule drugs which alter their ability to cross cell membranes are well-known. The ‘Rule of 5’ is a popular predictive tool for determination of a drug’s permeability *in vivo* based on its physicochemical attributes [52]. Solubility affects a drug’s partition coefficient [53], while molecular size and charge can similarly influence its movement [54]. The chemical properties of the ideal small-molecule drug are delicately balanced and it must be ensured that no one property is disproportionate. For instance, lipophilicity encourages drug movement across cell membranes, while hydrophilicity enhances drug diffusion around cells [55], and although it might seem that lipophilicity is

thus preferable for moving a drug inside a cell, an overly lipophilic compound is associated with a poor toxicological profile [56]. It is acknowledged that making an informed prediction of the path a compound is likely to take *in vivo* is highly complicated; thus, the use of various mathematical and computational techniques are advocated to combat this challenge [55]. The 'Rule of 5', for example, sets upper limits on a compound's lipophilicity (a logP no greater than 5) and molecular size (no more than 500 daltons) [54]. Additionally, physiologically-based pharmacokinetic modelling attempts to improve prediction accuracy by accounting for the variability of living systems [57].

There are several examples of BDEPT enzymes which introduce modifications to their prodrug substrates which disfavour their passage across cell membranes. Uracil phosphoribosyl transferase (UPRT), for instance, catalyses the conversion of uracil to uridine monophosphate, and converts 5-FU to 5-fluorouridine monophosphate which then disrupts RNA and DNA synthesis [58]. The activated form of 5-fluorouridine is phosphorylated and therefore its charge will impede membrane permeability. Similarly, thymidine kinase (TK) is a phosphotransferase that is utilised in BDEPT with the prodrugs ganciclovir (GCV) and acyclovir (ACV), phosphorylating them to inhibit DNA synthesis. This likewise results in a charged API, reducing transport across lipid membranes. However, phosphotransferase-based BDEPT has not proven to be ineffectual. *Bifidobacterium infantis* has been engineered to express TK derived from the herpes simplex virus type 1 (HSV-1), and significantly arrested the growth of murine bladder tumours following administration of GCV [59].

On the other hand, some enzymes can introduce favourable changes. For example, cytosine deaminase (CD) converts 5-fluorocytosine (5-FC) to 5-FU via the pyrimidine salvage pathway. 5-FU has lipophilic properties which are conducive to its movement across cell membranes. Following the first report of CD expression in *Bifidobacterium* longum [60], CD-BDEPT has since been tested preclinically with various bifidobacterial species. *B. longu*m expressing CD was used to localise prodrug conversion to autochthonous mammary tumours in rats, with corresponding antigenicity studies in guinea pigs confirming low immunogenicity [61]. *B. breve* was later found to more stably express the enzyme *in vitro*, showing 3- to 6-fold greater enzymatic activity than that of *B. longum* [62]. Non-bifidobacterial species have also been used to express CD. Preclinical *in vivo* studies in rhabdomyosarcoma-bearing rats showed that intratumoural injections of *Clostridium acetobutylicum* expressing CD, led to the conversion of 5-FC to 5-FU in the injected tumours [63].

Design Principle II: Orientation of the Enzyme

Bacteria, being living entities, may be subject to the same drawbacks of chemotherapeutic agents as human cells, including their accessibility to small-molecule compounds. This limitation can be addressed by manipulation of the enzymology of the BDEPT. The enzymatic reaction is the central event by which drugs are modified chemically in BDEPT. If chemical modifications of drugs bring about their activation, and impact their movement between extracellular and intracellular compartments, the designers of BDEPT must therefore understand the enzymology of their design, and how it could alter the pharmacodynamics and pharmacokinetics of a compound.

The biochemistry of GDEPT enzymes is known in detail [64, 65]. The design of GDEPT prodrugs and the pharmacology of their active forms are also well-known [66]. Despite the extensive range of enzymes used in GDEPT [67], the variety of BDEPT enzymes is comparatively low (Table 1). Presumably, this is because GDEPT, and its various offshoots such as antibody-directed enzyme prodrug therapy (ADEPT), are older styles of gene therapy [68]. It could also be the case that because many GDEPT enzymes are mammalian, without prokaryotic counterparts, their application in bacterial carriers is more complex.

Table 1. Examples of enzyme-prodrug strategies, their mechanisms of action, and the bacterial carriers with which they are associated.

Bacterial Carrier	Enzyme	Prodrug	Mechanism of Action	Reference
B. longum	**CD**	5-FC	Prevention of DNA synthesis by inhibition of multiple enzymes	[60, 61]
Clostridium sporogenes	**Nitroreductase (NTR)**	CB1954	DNA cross-linking	[69, 70]
VNP20009	**Carboxypeptidase G2 (CPG2)**	Range of aromatic nitrogen mustard prodrugs	DNA cross-linking	[71]
***E. coli* DH5α**	**β-glucuronidase (β-G)**	9-aminocamptothecin glucuronide (9-AG)	Topoisomerase inhibitor	[72]
Attenuated *Salmonella* SL7207	**TK**	GCV	DNA polymerase inhibitor	[73]
VNP20009	**Purine nucleoside phosphorylase (PNP)**	Fludarabine	Prevention of DNA synthesis by inhibition of multiple enzymes	[74]
Listeria monocytogenes	**CD/PNP/UPRT**	5-FC	Prevention of DNA synthesis by inhibition of multiple enzymes	[75]

The placement of the selected enzyme within a bacterial carrier is critical. If the enzyme requires reducing conditions or cofactors (e.g. NTR [76]), it should be expressed in the cytosol. However, a bacterium expressing a cytoplasmic, prodrug-modifying enzyme can activate a prodrug only if both are simultaneously present in the bacterial cell. An API without an aptitude for crossing the cell membrane may become trapped inside the bacterium after it is activated. If the prodrug itself is hydrophilic, it may not be able to enter the cell in the first place. This problem can be negotiated by tethering the enzyme to the cell wall of Gram-positive bacteria, or the outer membrane of Gram-negative bacteria using leader peptides and transmembrane sequence systems [12], or by re-routing it to the periplasm – a protein rich boundary found in the bacterial cell wall [77]. There are numerous examples of enzymes which have been subjected to this method in Gram-negative bacteria, including CPG2 and β-G (Figure 3).

CPG2 is a dimeric protein, derived from *Pseudomonas* sp. RS-16, that can remove a deactivating glutamate moiety from a range of mustard prodrugs [78]. This results in the formation of potent DNA cross-linking agents with high cytotoxicity differentials between drug and prodrug. Cytoplasmic CPG2 has not proven to be very efficacious, most likely because the prodrugs relevant to CPG2 are hydrophilic, and the enzyme naturally resides in the periplasm of *Pseudomonas* sp. RS-16. Periplasmic CPG2 has thus been investigated to confront this issue. CPG2 has been directed to the periplasm of VNP20009 via the *ompA* signal peptide, and was effective in activating many mustard prodrugs. This construct was tested with human breast and colon carcinoma cell lines, resulting in retarded tumour growth [71].

The hydrolytic activity of β-G is capable of rendering several drugs active, including phenol mustards and anthracyclines [79]. The enzyme has been experimentally expressed in *E. coli* DH5α cells, with results indicating inferior activation of irinotecan, a topoisomerase inhibitor, by the bacterial carrier compared with an adenoviral carrier expressing the same enzyme [80]. One of the reasons put forth to explain this observation was that the enzyme of *E. coli* DH5α was placed in its periplasmic space, and was therefore less accessible than the adenoviral enzyme which was delivered to the membranes of virally-infected cells. β-G has been directed to the outer membrane of *E. coli* BL21 by fusing it with an autotransporter protein, demonstrating activation of the p-hydroxy aniline mustard β-D-glucuronide and

inducing toxicity in experimental human colon cancer cells [81].

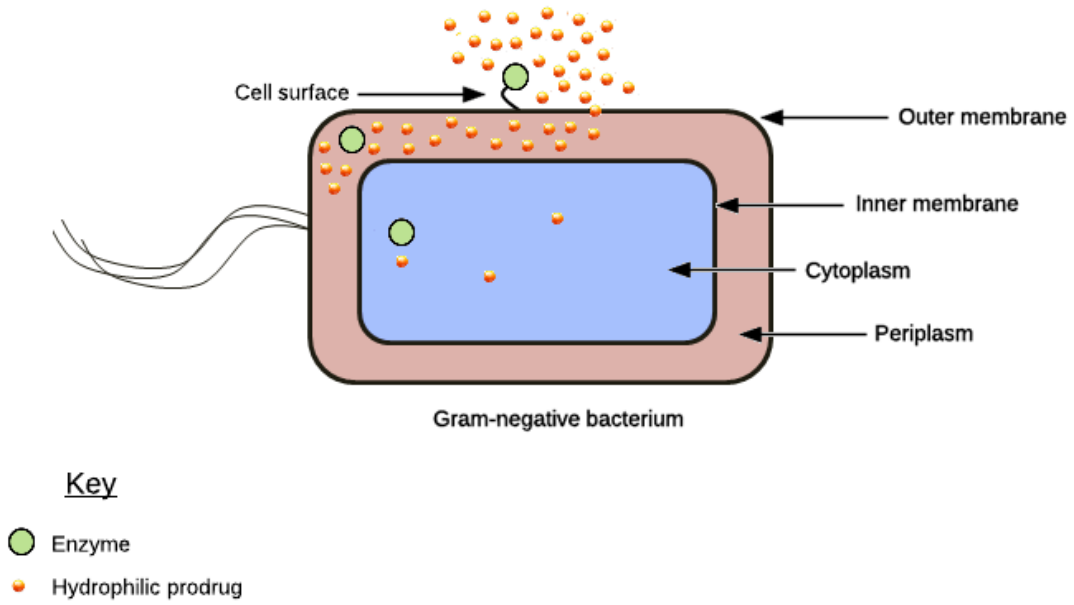

Figure 3. Enzymatic alignment strategies in BDEPT. *An illustration of the various enzymatic conformations that have been investigated in BDEPT. The activation of hydrophilic prodrugs has been enhanced by coupling BDEPT enzymes to the surface or directing them to the periplasm of Gram-negative bacteria (e.g. E. coli BL21 and VNP20009, respectively).*

Augmenting an enzyme's exposure to the prodrug by relocating it closer to the cell surface could possibly impact the stability of the enzyme itself. Some enzymes exhibit contrasting biochemistry when their expression is switched from cytoplasmic to periplasmic to surface-bound conformations. β-galactosidase, for example, is rendered toxic when exposed to the periplasm of *E. coli* [82]. On the other hand, the reduced quantity of proteases in the periplasm compared with the cytoplasm [83] may favour enzymatic stability in the environment of the former.

Design Principle III: Optimisation of Enzyme Kinetics

A central goal of BDEPT is to design a therapy with a high k_{cat} / K_M ratio. This ratio is commonly used to compare the kinetics of a single enzyme across multiple substrates, or to compare the kinetics of single substrate against multiple enzymes [84]. With BDEPT, both applications are relevant. Unmodified, the kinetics of several BDEPT enzymes leave much to

be desired and this requires reworking of either the enzyme or its substrate to ameliorate overall reactivity. Early efforts of enzyme optimisation include the mutagenic adjustments made to the HSV-1 TK [85]. Optimisation of this enzyme is needed because its unaltered form has a high K_M for ACV, and because GCV is immunosuppressive at therapeutic doses. A library of mutated TK enzymes was screened for improved kinetics, identifying three mutants which, when tested *in vitro* in a rat glioma C6 cell line, greatly increased the sensitivity of the cell line to GCV and ACV compared with native HSV-1 TK.

Enzyme kinetics are relevant also to CD. CD is not found in mammalian cells found but it is found naturally in the cytoplasm of bacteria [86]. Thus, bacteria which inhabit the human intestinal tract, and express the enzyme, can activate prodrug substrates with which they come into contact [87]. Wildtype CD has a relatively high K_M value for its prodrug substrate 5-FC than it does for cytosine [88], which warrants high doses of prodrug for BDEPT to be efficacious. This elicits undesirable, off-target toxicity.

The kinetics of CD have been revamped via mutagenic alterations. Amino acid substitution of regions lining the active site of CD has resulted in the identification of a CD variant with improved kinetics over the native enzyme in a xenograft tumour model [87]. Similarly, a shuttle plasmid incorporating CD with a mutation in its active site has demonstrated a 10-fold improvement in its kinetics over prior attempts [89]. Ideally, enhanced CD kinetics will help to overcome the impediments which are obstructing CD-BDEPT from the clinic, such as the rapid elimination of 5-FC from the systemic circulation and insufficient gene delivery.

The NTR family of enzymes has likewise been scrutinised for improved kinetics. NTR was among the first enzymes implicated in BDEPT, in conjunction with *Clostridium beijerinckii* [69, 90]. NTR activity is evident in anaerobic bacteria commonly found in the gastrointestinal tract [91]; thus, as with CD, prodrug substrates for NTR may cause non-specific toxicity when they are administered. The native NTR enzyme NfsB of *E. coli* can reduce the weak, alkylating 5-(aziridine-1-yl)-2,4-dinitrobenzamide prodrug CB1954 to potent DNA-intercalating agents, using NADH or NADPH as a cofactor [92]. NfsB was originally the most widely studied prodrug-activating NTR [93]. However, wildtype NfsB is kinetically unimpressive with multiple prodrugs [94, 95] and its high K_M has inspired research into alternative enzymes with better kinetic properties, such as NfsA. NfsA has demonstrated a higher k_{cat} / K_M ratio *in vitro* compared with NfsB for CB1954 [96].

As CB1954 is hepatotoxic when metabolised by the liver [97], setting tighter upper limits on doses of this prodrug is desirable. Other strains of bacteria, apart from *E. coli*, have been searched for the presence of NTR enzymes with improved kinetics that could allow dose reductions of CB1954. A fluorogenic probe was used to identify the flavin reductase MsuE, from *P. aeruginosa*, which exhibited a kinetically similar aptitude to the NfsA of *E. coli in vitro* for the reduction the next-generation DNA cross-linking 3,5-dinitrobenzamide-2-mustard prodrug PR-104A, and the next-generation nitrochloromethylbenzindoline prodrug CBI-DEI [98]. Furthermore, the NfsB enzyme of *P. aeruginosa* has recently been described for its improved kinetics *in vitro* over the NfsB enzyme of *E. coli* for reduction of CBI-DEI [95]. The newly-discovered enzyme NmeNTR, derived *Neisseria meningitidis*, has the advantage over the NfsB enzyme of *E. coli* in that it reduces CB1954 purely to the toxic 4-hydroxylamine derivative, whereas NfsB produces equimolar concentrations of the 4-hydroxylamine derivative, and the less toxic 2-hydroxylamine derivative [99].

Screening of NTR enzymes has led to the finding of more kinetically advanced NTR-BDEPT systems, which may allow dose reduction of CB1954. An oxioreductase library has been generated and screened using *E. coli* SOS reporter strains to quantify activation of CB1954 and PR-104A by NTR enzymes [100]. The *in vitro* sensitisation of *E. coli* cells to the activated prodrugs was used to evaluate enzyme efficiency. This led to the discovery of YcnD_Bs, which has a higher k_{cat} / K_M ratio than NfsA. It also led to the discovery of 14 reductases with higher k_{cat} / K_M ratios than NfsB_Ec, which up until that point was the most active PR-104A reductase.

As with CD, an effort has been made to forge mutated versions of NTR with better kinetics [101]. An *E. coli* SOS reporter strain was used to identify another flavin reductase, FRaseI from *Vibrio fischeri*, which displays better kinetics than NfsB *in vitro* in HCT-116 human colon carcinoma cells lines, and was further improved via site-directed mutagenesis [102]. Directed evolution, again using an *E. coli* SOS reporter system, has produced an NfsA variant with a higher affinity for PR-104A than native NfsA of *E. coli*, inducing higher drug sensitivities in human cell lines and mouse xenografts [103]. Moreover, a codon optimisation strategy was implemented with *C. sporogens* to heighten its ability to stably express NTR enzymes derived from *E.* coli [104]. Resultantly, doses of CB1954 and PR-104A, which were sub-therapeutic prior to codon optimisation, exhibited therapeutic potential thereafter in tumour-bearing mice.

Another approach to increase the k_{cat} / K_M of an enzyme is to manufacture better substrates for that enzyme. Novel prodrugs have been synthesised in the interest of developing better substrates for NTR. For example, 6-chloro-9-nitro-5-oxo-5Hbenzo(a)phenoxazine (CNOB) is a prodrug that can be converted by the enzyme ChrR6 to the cytotoxic and fluorescent agent 9-amino-6-chloro-5H-benzo(a)phenoxazine-5-one (MCHB) [105]. MCHB has been used with the non-virulent *Salmonella* strain SL7838 to treat murine tumours. Impressive remission rates were seen in mice implanted with tumours endogenously expressing ChrR6, following administration with CNOB. Unfortunately, only 10% of the mice treated with SL7838-ChrR6 survived after 60 days of treatment, implying that a defect was present in some facet of the BDEPT, possibly related to unsatisfactory availability of the bacterial enzyme to the prodrug.

Design Principle IV: Toxicity of the Prodrug/API to the Bacterium

Although BDEPT is designed to mitigate off-target toxicity to healthy tissues, the toxicology of the activated drug is still relevant. A chemotherapeutic drug which damages healthy and cancerous human tissue potentially poses a threat to the viability of bacterial cells. However, not all drugs will affect bacterial survival [106]. Studies with VNP20009 suggest that hydrophilic APIs are only harmful to the bacterium if, following activation outside the cell, the cell membrane is destabilised and the drug leaks into the bacterial cytoplasm [107]. If the drug is activated in the periplasm of the bacterium and is too hydrophilic to cross its inner membrane, the API will not harm bacterial DNA even if the enzyme is over-expressed.

The molecular differences between prokaryotic and eukaryotic cells are significant in that they may allow the mechanisms of action of chemotherapeutic cancer drugs to solely target mammalian cell machinery (the same principle holds in reverse to many antibacterial therapies, like penicillin, which relieve patients of unpleasant side effects by homing in on prokaryotic cellular peculiarities). The topoisomerase I inhibitor 9-aminocamptothecin (9-AC), for example, targets a topoisomerase enzyme chiefly expressed in mammalian cells [108, 109]. Hence, it should not pose a toxicological threat to bacteria. *E. coli* DH5α has been employed to deliver β-G to experimental tumours [72]. Here, the prodrug 9-ACG was administered, which is hydrolysed by β-G to 9-AC. Importantly, 9-AC did not destroy the bacterial vehicle, and *in vivo*, the combined treatment hampered CL1-5 human lung tumour growth in a murine model.

Some chemotherapies, however, can damage bacteria. A recent study catalogued the effects of various chemotherapeutic agents on lactic acid and bifidobacterial strains, indicating the sensitivity these strains have towards antimetabolite drugs like 5-FU and doxorubicin [110]. 2-fluoroadenine, a metabolite of fludarabine (Figure 4), has antibacterial properties [111]. PNP, the enzyme which catalyses the conversion of fludarabine, can break down non-toxic deoxyadenosine molecules into highly toxic adenine analogues [112]. The endogenous PNP enzyme of *Salmonella* (genetically homologous to the PNP of *E. coli*) has been exploited to convert 6-methylpurine-2-deoxyriboside (6MeP-dR), a substrate for bacterial PNP but not human PNP, to 6-methylpurine (6MeP). *In vivo*, murine B16F10 tumour growth was diminished following this regimen [74]. Though the inhibition of DNA synthesis instigated by these toxic molecules results in tumour cell death, bacterial viability too may be affected. This may partly explain why adenoviral PNP delivery has enjoyed success in phase I clinical trials [113], while PNP-BDEPT has yet to be tested in patients.

Our studies of CB1954 revealed that bifidobacterial strains and certain *E. coli* strains (e.g. MG1655) are highly sensitive to the prodrug, whereas other *E. coli* strains (e.g. *E. coli* Nissle 1917) and *Lactococcus* are more resistant. Western blot studies of NTR suggest that high concentrations of NfsaA and NfsB are responsible for the toxicity of CB1954 to bacteria. The activated forms of CB1954 are a mixture of 2- and 4-hydroxylamines, of which the latter is the more effective DNA cross-linker (Figure 4). The hydroxylamine compounds to which CB1954 is metabolised are mutagenic and are inhibitory to bacterial DNA, RNA, and protein synthesis [114, 115].

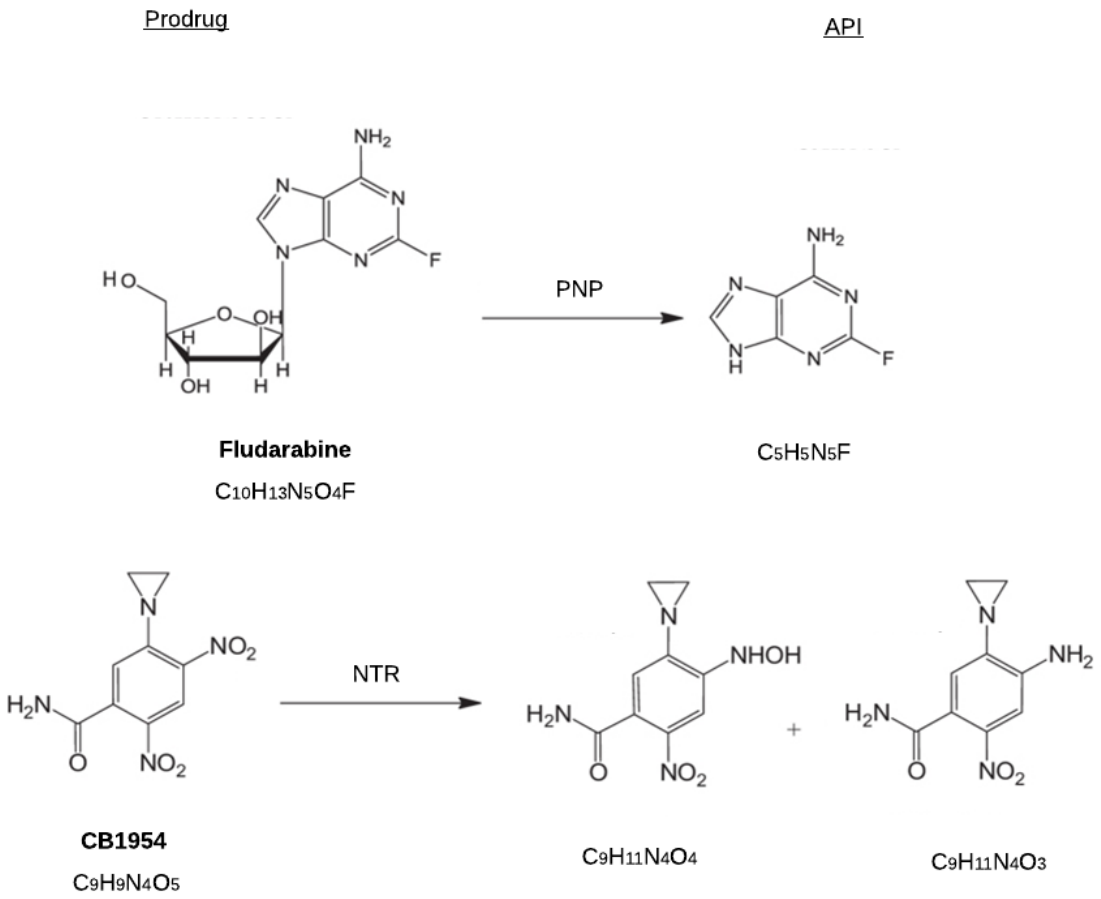

Figure 4. Toxic metabolite production via BDEPT enzymes. *Fludarabine is converted by PNP to 2-fluoroadenine ($C_5H_5N_5F$), while CB1954 is converted by NTR to DNA cross-linking agents, all of which are potentially deleterious to bacteria via inhibition of DNA, RNA, and protein synthesis.*

Toxicity issues can be worked against by enzymological adjustment of a BDEPT. While it is a feasible tactic for enriching the activation of a hydrophilic prodrug, re-orientation of an enzyme such that it is closer to the surface of the bacterial cell can also be applied to circumvent toxicological threats to bacteria. Again, the implications of engineering the enzyme and possibly affecting its activity, as well as the distribution pharmacokinetics of the API, should be borne in mind. A lipophilic, toxic API, despite being activated in a non-cytoplasmic region, may nevertheless cross into the cytoplasm of the bacterial cell, exerting a toxic effect. It is vital at this point for the designer to understand both the toxicity and

membrane permeability of their compound, because this will clarify whether re-calibration of the enzyme will be truly beneficial to BDEPT efficacy

Design Principle V: Choosing a Bacterial Carrier

At this stage, having defined the pharmacological and toxicological traits of both drug and prodrug, and having gained an appreciation of the biochemistry of their chosen enzyme, the designer should be able to deliberate on an appropriate bacterial carrier. This decision should integrate four separate considerations: the level of invasiveness desired of the bacterium, the appropriateness of a wildtype carrier versus a recombinant carrier, the advantages offered by Gram-positive versus Gram-negative bacteria, and facultative versus obligate anaerobic bacteria.

(i) Intracellular vs. Extracellular Bacterial Carriers

In BDEPT, the bacterium must be positioned such that it is capable of enzymatically activating its prodrug target. If prodrug metabolism is to occur within the tumour cell, the bacterium will require intracellular access to supply its enzyme, but the requirement is waived if the drug can enter the cancer cell following activation. 'Bactofection' is the bacterial equivalent of transfection, whereby an invasive bacterium is engineered with a plasmid featuring a transgene under the control of a mammalian promoter which, following administration, invades the host, lyses, and releases the plasmid within the host's cells [116]. The plasmid migrates to the host cell's nucleus where the transgene can be transcribed. This procedure typically relies on disease-causing bacterial strains, presenting safety concerns in terms of off-target toxicity and immune stimulation [19]. An example of bactofection involves exploitation of attenuated *L. monocytogenes* administered to murine tumours *in vivo*, and human breast tumour tissues *ex vivo* [116]. The microbial cells were sensitive to ampicillin and were ruptured following systemic administration of the antibiotic. This caused the release of plasmid DNA, and the subsequent expression of a lysin transcribed by a mammalian promoter. Additional cell lysis occurred due to heightened expression of the lysin itself.

In BDEPT, bactofection can be applied if the API has difficulty crossing cell membranes, if the enzyme cannot be expressed inside bacterial cells optimally, or in a 'host cell conditional' strategy involving expression of genes from a mammalian promoter. Non-invasive and non-

pathogenic bacteria have been engineered to confer upon them invasive qualities. For example, the bacterial designer strain *Escherichia coli* BM2710 was engineered to express two genes to formulate an invasive strain: the *inv* gene from *Yersinia pseudotuberculosis* which facilitates invasion of mammalian cells that express the β1-integrin, and the *hly* gene from *Listeria monocytogenes*, which induces phagosomal escape and the release of bacterial cytoplasmic proteins and plasmid DNA into the cytoplasm of mammalian cells [117].

Bactofection has been incorporated into several BDEPT designs to internalise drug activation in the target cell. This overcomes the drawback of enzymes like PNP and TK, which phosphorylate their targets, thereby impeding their movement into cells. *L. monocytogenes*-mediated bactofection has delivered PNP into several tumour cell lines, allowing efficient cytotoxicity [75], while attenuated *Salmonella* has been employed to deliver TK to experimental B16 melanoma cells *in vivo* in which, following GCV administration, significant suppression of tumour growth was observed [73].

Anticancer therapies which rely on intracellular agents to deliver genes to tumours generally fall drastically short of depositing their payload in the entirety of the malignancy [9, 118]. The bystander effect is critically important for these intracellular agents. The bystander effect hinges on the idea that tumour cells which do not acquire the gene of interest may nevertheless be affected by downstream events resulting from its expression in other cells. Within the context of BDEPT, these downstream events refer to the passage of prodrugs into tumour cells containing the gene of interest, and the passive or active translocation of cytotoxic metabolites into local cells or distant tumours [67]. An understanding of the bystander effect is an essential part of predicting the efficacy of BDEPT mediated by intracellular bacteria. The importance of the bystander effect is evident in a study in which an attenuated strain of *S. typhimurium* was used to deliver PNP *in vitro* to murine Lewis lung carcinoma and melanoma cells [119]. Significant reductions in cell survival were observed, even in cases where only 1% of cells tested positive for the carrier.

The impact of the bystander effect can be magnified, often by the use of kinetically-superior, mutated [87] or naturally-occurring enzymes [96], or alternative prodrugs [95, 120]. The *in vitro* measurement of the bystander effect is sensitive to the type of cell line analysed [118]. Multilayer and spheroid cell lines are advocated for their stronger resemblance to the tissue densities of tumours compared with monolayer cell lines. Most importantly, the bystander

effect is intertwined with therapeutic efficacy; thus, an increase in its magnitude will permit dose reductions of administered prodrugs.

(ii) Recombinant vs. Wildtype Bacterial Carriers

It is recommended that bacterial recombination of enzyme-coding genes should take place within chromosomal DNA rather than within plasmid DNA [99]. Not only does chromosomal integration increase the stability of transgene expression, but chromosomes are not considered 'mobile genetic elements', and are not known to partake in horizontal gene transfer from one bacterium to another, unlike plasmids [121]. In this way, translocation of genetically-engineered material between microorganisms *in vivo* is reduced, which is of great significance to the regulators of recombinant investigational medicinal products (see later).

Bacteria that have been engineered to express heterologous genes at high levels can synthesise enzymes in large quantities [122]. However, recombinant bacteria are liable to suffer from a metabolic burden resulting from gene overexpression. This burden originates from a divergence of the microbe's cellular resources so that transgene expression can be maintained. This can overload the carrier and disrupt its targeting capacity due to loss of its transgene and compromised viability [123]. Transgene expression can be negated due to spontaneous loss of plasmids [124, 125]. In this case, the survival of genetically-unencumbered microbes which have lost their transgene is favoured, while recombinant bacteria are eventually eliminated by natural selection.

If recombinant gene overexpression is a potential threat to the integrity of the bacterial carrier, this problem could be alleviated by exercising stricter control over bacterial enzyme production. Burdensome recombinant gene expression reduces the pliability of BDEPT carriers, as it complicates the production of appropriate levels of enzyme. *E. coli*, for instance, possesses natural proteolytic facilities to counteract the overexpression of proteins which do not benefit its survival [126]. This problem can be manoeuvred by using protease-deficient or heat shock-deficient strains, for example. An excessively taxed bacterium, deprived of amino acids due to transgene expression, could also be supplemented with exogenous amino acids to correct the depletion.

Putting genetically-engineered, recombinant bacteria aside, the BDEPT designer could also entertain the option of using wildtype bacteria which naturally express prodrug-converting

enzymes. Wildtype bacterial cells, by definition, tax themselves according to what is required by their default cellular needs, and are not expected to mobilise additional resources to produce foreign biomolecules. This means that the expression of prodrug-converting enzymes by wildtype bacteria could overstep the disadvantages of using recombinant strains, because natural expression would be less likely to affect the integrity of the carrier. Wildtype bacteria have been used to activate different prodrugs. Our laboratory has successfully employed the prodrug fludarabine in conjunction with the endogenous activity of *E. coli* Nissle 1917 to induce cancer cell death in multiple cell lines [106, 127]. We have reported that several species of bacteria can activate CB1954 with a natural enzymatic complement, and have shown that it is possible to activate multiple prodrugs concurrently (5-FC, CB1954 and fludarabine), presenting opportunities for treating refractory tumours with more than one prodrug. Interestingly, prodrugs can be activated without a known corresponding enzyme, suggesting that wildtype bacteria have an 'enzymolome' that could be harnessed to activate drugs for cancer therapy. For instance, the drug AQ4N can be activated by *E. coli* Nissle 1917 *in vitro* to kill cancer cells, although the mechanism of activation is unknown.

While the notion of delivering a carrier with a diverse enzyme reservoir to a tumour appears auspicious in terms of prodrug activation and therapeutic efficacy, our lab has detailed a range of prodrugs frequently used in mainstream chemotherapy that can be deactivated by bacterial enzymes, including etoposide phosphate, cladribine, and gemcitabine [127]. This is not unexpected as a bacterium's enzymolome is vast and may harbour enzymes which deactivate certain drugs while activating others. This clinically relevant phenomenon ought to be addressed by the designer irrespective of whether they integrate a recombinant or wildtype strain into their design.

(iii) Gram-Positive vs. Gram-Negative Bacterial Carriers

When selecting a bacterial carrier, it is helpful for the designer to contemplate the influence, both physical and biochemical, of inner and outer bacterial membranes on prodrug activation. This necessitates discussion of Gram-positive and Gram-negative bacteria, which differ from each other in the constitution of their cell envelopes. The cytoplasm of both Gram-negative and Gram-positive bacteria is separated from the extracellular environment by a cell envelope. In Gram-negative bacteria, this consists of an outer membrane and an inner membrane, separated by a cell wall containing the periplasmic space [77]. Most importantly,

in Gram-positive bacteria, an outer membrane is lacking, and the existence of a Gram-positive periplasm is less noted, although it has been described [128-130]. This should be taken into consideration when mapping-out a BDEPT design. The differences between Gram-positive and Gram-negative cell envelopes have implications for bacteria in terms of the way they interact with their host, and their flexibility as a BDEPT carrier.

Examinations of VNP20009 have revealed the affliction that Gram-negative bacteria can visit upon their host. Lipid A, which forms part of the lipopolysaccharide (LPS) component of the outer membrane, can provoke toxic shock syndrome in the organisms they inhabit [131]. A convenient workaround for this obstacle involves deleting the bacterial gene *msbB*, which abolishes the toxicity of lipid A [132, 133]. LPS plays another role by decreasing the permeability of the outer bacterial membrane, although mutations in *msbB* do not appear to affect the structural integrity of this barrier [134].

With Gram-negative bacteria, the outer membrane represents a supplementary blockade against the admission and escape of prodrug and API to and from the cell, limiting the passage of large, charged molecules, but letting hydrophilic molecules in through porin channels [135]. Lipophilic molecules are less prone to crossing the outer membrane than the inner membrane, probably because of the comparatively disordered arrangement of the inner membrane's lipid content [136]. Overall, the multi-layered, multi-functional barrier that is unique to Gram-negative bacteria complicates the migration of both hydrophilic and lipophilic drug molecules due to the inner and outer bacterial membranes, respectively.

(iv) Oxygen Tolerance of Bacteria

Differences in oxygenation between tumour tissue and healthy tissue, and even between different parts of the tumour itself [137], is a foundational aspect of tumour-selective colonisation by bacteria. Selectivity occurs because the hypoxia that is evident in tumours does not materialise as severely in healthy tissues [20]. The selective colonisation of necrotic, hypoxic tissue by anaerobic bacteria is viewed as advantageous, because it permits access to parts of tumour tissue that are notoriously hard to penetrate using standard chemo- and radiotherapy [47].

There are drawbacks of exclusive necrotic tissue colonisation that ought to be noted. An obligate anaerobe that colonises only necrotic tissue will be farther removed from incoming

prodrugs which are chiefly delivered to oxygenated tissues [137]. Alternatively, a flexible facultative anaerobic bacterium which can acclimatise to varying grades of oxygenation will be better placed than an obligate anaerobe to promote maximal prodrug activation at its intended site of action. One might expect this flexibility to come at the price of potential colonisation of more oxygenated extra-tumoural tissues and consequential systemic toxicity, however both obligate and facultative anaerobic bacteria display similar tumour-to-liver colonisation ratios [32, 138].

While facultative anaerobic bacteria are, in theory, more likely to inhabit more diverse tumour regions than their obligate anaerobic brethren, their adaptability is not assured. *S. typhimurium*, for example, has been shown to colonise only necrotic regions of murine tumours, despite being a facultative anaerobe [35]. An auxotrophic variant of this same bacterium has since been engineered and observed to grow in both viable and necrotic tissue [30]. It is important to be cognisant that different auxotrophic modifications can be used to influence such bacterial platforms in different ways; for example, in the case here the mutation increases the range of tumour tissue within which the bacterium can grow (due to oxygen-related metabolic activity) while in other cases, auxotrophic mutations restrict where a bacterium may survive.

Improving BDEPT R&D with Tools for Monitoring Bacterial Enzymatic Activity

The defining reason for failure of oncological medicines in late stage clinical trials is a lack of therapeutic efficacy [139]. One of the causative factors may include insufficiencies in the technologies available for therapeutic drug monitoring *in vivo*. These technologies could help familiarise investigators with the *in vivo* agency of elusive, novel medicines like BDEPT. For gene therapies in particular, the Recombinant DNA Advisory Committee of the National Institutes of Health recognises the need for reliable assays of transgene expression, and quality control of gene therapy carrier trafficking [140]. Blood sampling and tissue biopsies are invasive, periodic, and retrospective, and introduce convolution into the procedure [141]. Contemporary standards therefore demand non-invasive, real-time facilities for continuous assessment of transgene expression and therapeutic efficacy. The availability of a high-calibre facility for monitoring the progress of a medical intervention is correlated with an accelerated bench-to-bedside timeframe [142], so it is worthwhile reflecting on how BDEPT can be satisfactorily monitored to mobilise its translation into the clinic.

For the BDEPT designer, real-time monitoring of the impact of the bacterial carrier and prodrug administration on tumour regression is an invaluable way of evaluating responsiveness to a medical intervention. The unique features of BDEPT offer the designer a special and convenient vantage point for surveillance of the course of treatment. The selective colonisation of tumour tissue by bacteria, and the resultant enzyme expression, can be utilised to fashion a prodrug-like compound into an imaging probe. In this setting, the probe is attached to a signal quencher which, when enzymatically cleaved, emits a detectable signal which can be observed *in vivo* using imaging techniques. Thus, a prodrug which is visually detectable when activated by a bacterial enzyme is indicative of transgene expression. A further potential benefit of this strategy involves the generation of data indicating the location and size of a malignancy.

Readouts can be produced via molecular imaging (MI) techniques, such as optical imaging (OI), positron emission tomography (PET), and magnetic resonance imaging (MRI) [143]. For preclinical imaging, OI represents the most feasible method for researchers in terms of accessibility, however, PET and MRI are more informative approaches for determination of clinical activity, and aid the formation of a complete diagnostic picture [144, 145]. Several imaging modalities exist for non-invasive visualisation of bacterial enzymatic activity [36, 146, 147]. Critical proof-of-concept issues can be tackled by adoption of these technologies, including quantification of bacterial growth, assessment of intratumoural growth patterns, and confirming whether tumour-specific growth has occurred at all.

Various dyes exist which are reduced to their fluorescent form by NTR. These include the redshifted, quenched fluorophore, CytoCy5S, which is reduced to its fluorescent form by NTR [148, 149]. We have shown this probe to be useful in tracking the location of tumour-targeting bacteria *in vitro* and *in vivo*. [150, 151] VNP20009, expressing TK, has been engineered to enable its visualisation in C38 colon and B16-F10 melanoma tumours in mice [152, 153]. The radiolabelled nucleoside analogue 2'-fluoro-1-beta-D-arabino-furanosyl-5-iodo-uracil (FIAU), following VNP20009-TK-mediated phosphorylation to sequester it within tumours cells, allowed visualisation of bacteria *in vivo* by PET and autoradiography. Bacterial proliferation in tumours could be easily monitored, as radioactivity levels correlated with the numbers of bacteria present. Similarly, CPG2 activity has been monitored *in vivo*, in a xenograft murine tumour model, using the ^{19}F MRS reporter molecule 3,5-DFBGlu [154].

While CytoCy5S, FIAU, and 3,5-DFBGlu are advantageous in that they permit monitoring of enzymatic activity, they are not therapeutic with respect to tumour growth inhibition. Several compounds have been synthesised which function as both therapeutic and monitoring agents. CNOB, for example, is converted into MCHB, which is both fluorescent and cytotoxic [105]. Moreover, the conversion of ^{19}F-labelled 5-FC into cytostatic 5-FU by VNP20047 expressing CD as a reporter gene has enabled visualisation of murine tumours *in vivo* in a human HCT116 colon tumour xenograft by magnetic resonance spectroscopy (MRS) [155].

BDEPT enzyme activity may also be examined in *ex vivo* samples through indirect methods which assess for the presence of activated probes within the sample (Figure 5). Such strategies have also been proposed as potential cancer diagnostics, in that they may reveal the presence of a tumour within the body due to their acting as a readout for administered bacteria which have colonised malignant tissue. For example, *E. coli* expressing a regulated *lacZ* gene was designed to detect experimental liver metastases [156]. Following bacterial colonisation of hepatic tumours in mice, the bacterial *lacZ* device was induced by systemically administered isopropyl β-D-1-thiogalactopyranoside. Subsequently, a caged luciferin probe was administered, cleaved, and activated by lacZ, and renally-excreted. Urine samples were quantified by emission of light for the presence of luciferin, facilitating quick and non-invasive cancer detection. In a similar fashion, Panteli *et al.* created an inducible biomarker module that can be detected in blood samples by antibodies using a type of enzyme-linked immunosorbent assay [157]. The biomarker, ZsGreen, was expressed by *Salmonella* in a preclinical model to detect experimental colon carcinoma tumours.

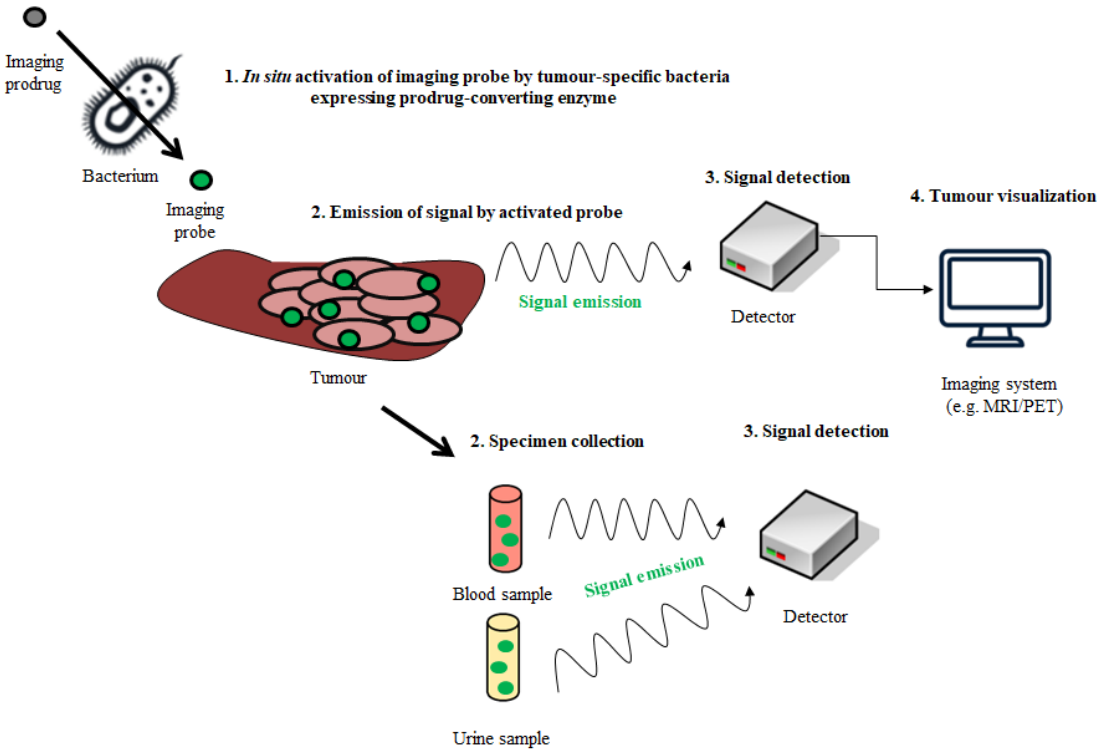

Figure 5. BDEPT enzymatic activity detection. *Therapeutic enzymatic activity may be detected in situ, or ex vivo. Direct in vivo detection can be accomplished by activation of a light-emitting probe by bacterially-expressed enzymes at the site of the tumour, followed by MI to acquire an image of the probe. Indirect detection may be achieved by analysis of blood or urine samples for probes that have been activated by bacterial enzymes in the tumour and secreted into the circulation.*

Barriers to the Clinic

Several examples of clinically-trialled BDEPT exist; however, the regulatory requirements necessary to make a breakthrough, regulated medicine of this kind have yet to be satisfied. It may be insightful to review the clinical progress achieved thus far with this therapy. The reasons for the absence of BDEPT from the clinic are probably multi-faceted, and the way in which designers comprehend and confront the barriers to the clinic will dictate how readily access to it is granted.

CD-BDEPT has been tested in phase I clinical trials with VNP20009 [25]. Data were collected for 25 patients – 24 patients with melanoma, and one patient with metastatic renal cell carcinoma. Despite using an attenuated strain, doses above 3×10^8 colony-forming units/m^2 resulted in low levels of tolerance in patients, and only 3 patients had tumours in which the administered bacterium was detected. Tumour regression did not occur in any

patient. Trial investigators noted that preclinical studies in mice were not reflective of what was witnessed clinically, and speculated about host-specific differences between murine and human tumour composition that could affect colonisation of bacteria in malignant tissue. Following publication of the data for the phase I clinical trial, an update was issued describing the administration VNP20009 in four additional melanoma patients [27]. Results mirrored those of the pivotal study, with no patient achieving a significant clinical response.

After the phase I clinical trial of VNP20009, a pilot clinical study of CD-BDEPT was conducted with VNP20009. This was narrower in scope than the pivotal study, recruiting three patients with head and neck cancer. [158]. Notably, the gene for CD was chromosomally integrated into VNP20009 to increase therapeutic efficacy. Intratumoural injections of VNP20009-CD resulted in persistence of bacteria in two of these patients, bringing about the conversion of 5-FC to 5-FU at the patients' tumour sites. No significant measurements of 5-FU were made systemically. Even so, no tumour regression was observed in either patient and the trial was discontinued. This was a disheartening conclusion to encouraging preclinical data. King *et al.* had previously demonstrated efficacy in murine tumours using VNP20009 harbouring CD in which an 88-96% inhibition of tumour growth was observed in mice treated with VNP20009-CD and 5-FC compared with 38%-79% inhibition achieved with VNP20009-CD alone [159].

The results of the pilot and pivotal clinical trials of VNP20009-CD call for an explanation as to why tumour colonisation patterns differ between murine models and human subjects. Taken together, two factors appear to be common to both studies: poor enzymatic activation of 5-FC and poor colonisation of tumour tissue by VNP20009. It cannot be overlooked that the lacklustre kinetics of wildtype CD, referred to earlier, may explain why human trials of VNP20009-CD have so far returned with disappointing results in terms of inadequate tumour regression in tumour-colonised patients. Furthermore, as mentioned previously, colonisation of VNP20009 within tumours tends to be compartmentalised within necrotic regions, which are distinct from the viable zones of tumour tissue into which prodrugs are primarily delivered. A review of the literature reveals various efforts which endeavour to address these problems. The identification of auxotrophic VNP20009 mutants and more efficient CD variants, referenced above, are clear attempts to improve tumour colonisation and tumour growth inhibition, while reducing the bacterial burden on the patients to whom the therapy is administered.

Recently, a transformed strain of *B. longum* (APS001F), containing a point mutation in its CD gene, was described as having 10-fold greater enzymatic activity *in vitro* than its wildtype counterpart [160]. It subsequently demonstrated significant inhibition of tumour growth in murine models following 5-FC administration, and is the subject of a phase I/II clinical trial presently enrolling oncology patients (clinicaltrials.gov identifier: NCT01562626). Results from this trial are eagerly awaited, and may shed light on the effects of enzyme optimisation on the therapeutic efficacy of BDEPT.

Beyond therapy-associated parameters potentially responsible for inconsistencies between clinical and preclinical findings, external causes of disparities could be traced to the properties of the animal model used. A 'quick win, fast fail' approach is advised in research and development (R&D), by which the viability of a product is determined at the earliest possible stage [161]. Acquiring insights into product inadequacies at the preclinical stage allows for expedited curtailment of failing R&D projects, thereby improving R&D productivity. Of course, the choice of an appropriate preclinical model which is the most illustrative of the clinical reality is an indispensable part of this process.

There has been a recent reallocation of interest to non-murine animal models, such as canine models, which may be more representative of a human response to cancer treatment [162, 163]. The use of naturally-occurring tumours in companion dogs has been examined for the benefits it offers over artificially-induced tumours in mice for predicting clinical response. The genetically heterogenous character of spontaneous tumours in canines keeps more closely to the disparate nature of human malignancies than the artificial tumours of inbred mice, which have more a more homogenous make-up. Additionally, the circulatory system of dogs more closely resembles that of humans in terms of blood volume. The more expansive blood supplies of humans, compared with mice, can reduce bacterial colonisation within tumours. Tumours themselves within larger animals also tend to be comparatively smaller.

The higher financial costs of carrying out canine tumour experiments compared with murine experiments is apparent [162]; however, dose-finding studies in mice may underestimate the dose necessary to achieve tumour colonisation in humans, due to incompatible physiologies. This may lead to more dissatisfactory patient responses in the clinical setting, ultimately leading to greater losses of time and money overall. Indeed, a canine study which compared intravenous and intratumoural administration with *C. novyi*-NT reported a significant anti-tumour response when the bacterium was administered directly into the tumour, with no

animal achieving a complete response when administration was intravenous [50]. Had such a study been conducted in mice, it is possible that intravenous administration may have been sufficient for tumour colonisation, giving perhaps a more distorted reflection of what might occur in human subjects. A further study in dogs injected intravenously with *C. novyi*-NT spores reported a high frequency of noxious events resulting from treatment [164].

Gene therapy medicinal products are increasingly navigating their way into clinical trials, overseen by regulatory authorities which deliberate on whether the product makes it from one stage to the next. Regulatory authorities set stringent criteria against which the safety and efficacy of medicinal products are judged. This applies, too, to gene therapies, for which the use of live carriers to transmit therapeutic material warrants special regulatory consideration. Therapies containing live bacteria, up until now, have had a relatively weak foothold in the clinical armamentarium. Bacillus Calmette-Guerin (BCG), a treatment for non-muscle invasive bladder cancer, is the only live bacterial therapy that is currently approved by the Food and Drug Administration [165], and apart from being attenuated, is not genetically-engineered [166, 167]. Safety assurance is key with bacterial treatments. Microbiological testing is a fundamental part of sound safety assessment of cancer gene therapies, and contamination of bacterial therapies with fungi, or indeed other bacteria, is a pressing regulatory concern [168]. In fact, recent shortages of BCG have been attributed to possible fungal contamination [169]. This underlines the importance of quality control and quality assurance in the manufacture of therapies like BDEPT.

Genetically-engineered bacteria intended for *in vivo* applications face an atypical kind of regulatory hurdle in terms of safety assurance. The transference of resistance genes from genetically-engineered bacterial therapies to host microbiota and pathogenic agents could result in the genesis of antibiotic-resistant disease, compromising patient safety. Thus, bacteria must be rid of antibiotic resistance genes before administration to clinical trial subjects can take place. Even innocuous probiotic bacteria, when formulated as feed additives, must be devoid of antibiotic resistance genes before they can be approved for use in Europe [170]. Microbes, even those which have been genetically weakened, are at risk of being shed from the initial host and spread from person-to-person [168]. Thus, antibiotic-resistant disease could manifest at both the intra- and inter-individual level. Bacteria should ideally be disabled from replication in the final product, and in case of their release into the environment, where they could be easily acquired by other individuals, their growth should be negated. Heap *et al.* have recently developed a technology which obviates the need for

inclusion of antibiotic selection markers in bacterial strains [171]. They thereafter reported the first example of a bacterium, *C. sporogenes*, with a chromosomally-encoded prodrug-converting enzyme that does not require antibiotic resistance genes [99]. The strain was also rendered auxotrophic by inhibiting its ability to synthesise pyrimidines, meaning that it would not proliferate if it were released into the environment. In doing so, they have invented a strain that potentially has a far more attractive safety profile for patients, which could help further ease regulators' worries about the propagation of antibiotic resistance.

BDEPT Prospects

The ingredients necessary for transportation of BDEPT into the clinic may already be available, but their correct management and coordination by the designer will prove instrumental in building a meaningful product. As the bacterial carrier is the defining feature of this type of DEPT, a bacteria-centric focus stands to illuminate the way forward in terms of tackling deficiencies in BDEPT designs.

There is much to learn from the technical shortcomings of recent preclinical investigations of BDEPT that can be instructive for prospective design attempts. It has been reported that lesser prodrug activation using BDEPT, compared with VDEPT, could be accounted for by disparities between viral and bacterial carriers which lead them to concentrate in different types of tissue [80]. Viruses, such as those derived from the murine leukaemia virus, gather only in viable, proliferating cells [18], while bacteria colonise both viable and necrotic tissues to different degrees, depending on their ability to thrive in oxygenated and deoxygenated environments. It may be constructive to bear this in mind at the design stage. The ideal bacterial carrier will colonise diverse types of tumour tissue. Poor colonisation of viable cells will diminish prodrug activation, while poor colonisation of hypoxic, necrotic tissue may affect therapeutic potential to reduce tumour growth. It would be prudent to clarify the intratumoural growth patterns of the bacterial carrier prior to its inclusion in the BDEPT design.

Other bacterial properties which merit further investigation include the bacterial cell envelope. The Gram-positive periplasm is smaller than that of Gram-negative bacteria [172], which may explain why only the Gram-negative periplasm has been exploited in BDEPT to improve enzymatic activity; however, an unfamiliarity with the Gram-positive periplasm in general could be limitational. Given that Gram-positive bacterial cell envelopes omit an

obstructive outer membrane, an enzyme incorporated into the Gram-positive periplasm will be more accessible to a circulating prodrug. The Gram-negative bacterial periplasm, although more familiar to the designer, is sheathed in greater membranous complexity. It may therefore be productive for BDEPT designers to investigate the use of the Gram-positive periplasm as a means of improving BDEPT efficacy.

Both Gram-positive and Gram-negative bacteria contain membrane-associated efflux pumps which can export drugs from the bacterium [173, 174]. Efflux pumps are conventionally studied in the context of multidrug bacterial resistance against antibiotics, so not much is known about them in relation to chemotherapies. Nonetheless, there is speculation that they may be responsible for ejecting chemotherapeutic drugs from bacteria [110]. While this may have a hand in contributing to bacterial resistance to chemotherapeutic toxicity, efflux pumps could be seen alternatively as passageways through which an API may make its way outside a cell. Future BDEPT designs might benefit from considering the potential for efflux pumps to act as escape hatches for drugs which would otherwise be trapped by their unfavourable pharmacokinetics. Research into the effects of bacterial efflux pumps on chemotherapeutic drugs would be welcomed in this regard.

Stimulating productivity in R&D is contingent on researchers keeping abreast of emerging technologies that could help expedite their products into the clinic. Synthetic biology is a novel scientific discipline which offers a profound design tool, giving its practitioners the means to bioengineer molecular circuits, enabling the design of ‘intelligent’ cellular systems [175]. It is a valuable discipline from a BDEPT perspective, because it allows its designers to assemble ‘parts’ (e.g. promoters and secretion tags) into ‘devices’ (e.g. a plasmid or chromosome) within host systems called ‘chassis’ (i.e. the bacterial carrier), permitting fine-tuning of enzyme expression and function (Figure 6). Enzymatic constants like K_M and k_{cat} are relatively easy to predict. However, these constants change inside the chassis, making it difficult to calculate the kinetics of the enzyme. Synthetic biology can be utilised to fine-tune the chassis and the device together for optimal performance.

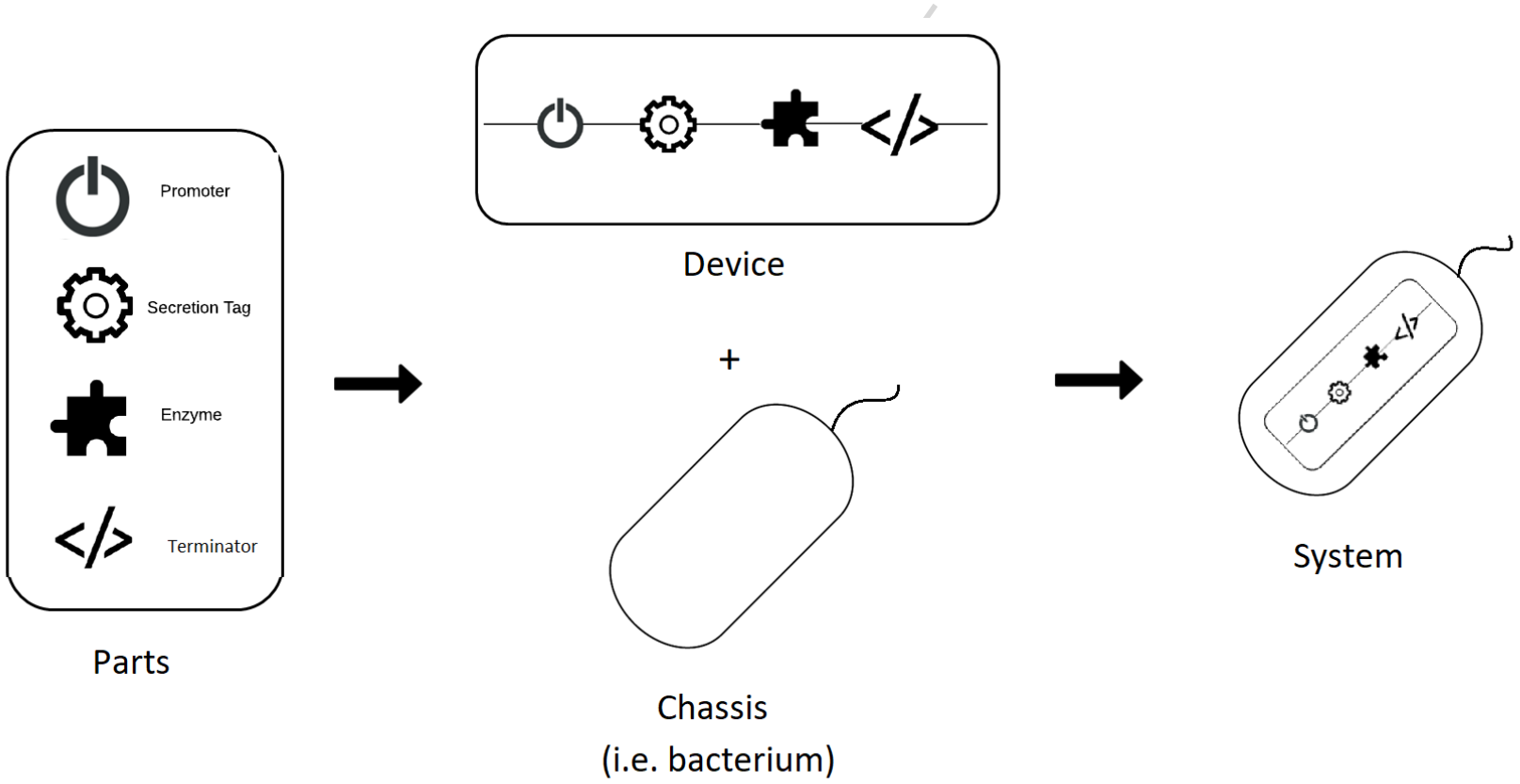

Figure 6. The application of synthetic biology to BDEPT. *Synthetic biology rationalises BDEPT systems design by reducing its holistic structure to well-characterised, functional "parts" which, in concert with one another, are built into a modular "device" to carry out a predefined role. The device, when embedded in a chassis, forms a "system" which is fit to complete a task in accordance with its design. Within BDEPT, bacteria act as chassis for devices in the form of plasmids and chromosomes. These devices are equipped with various parts, e.g. secretion tags, which aim to coordinate the expression of a prodrug-converting enzyme.*

Synthetic biology has been investigated for its applicability in perfecting bacterial homing to tumours. While bacteria are indeed tumour-selective, clinical experience thus far underlines the reality that human tolerance of administered bacterial loads places restrictions on BDEPT dosing regimens [25, 27]. In fact, there are instances where even commensal bacteria have had dosage limitations placed upon them in preclinical studies [32]. From synthetic biology principles, synthetic adhesins have been assembled based on the β-barrel domain of intimin - an adhesin derived from pathogenic *E. coli* strains - and the variable immunoglobulin domain of heavy chain-only antibodies [176]. When affixed to bacteria, these adhesins raise the tumour-targeting capacity of the chassis, and reduce the bacterial titre required for tumour colonisation.

Gene therapies in general have had scattered clinical fortune, and even those which are therapeutically efficacious and safe in clinical trials have ultimately been unusable due to commercial incompatibilities [177]. This means that BDEPT can undergo translation from bench-to-bedside, and its developers are still liable to be short-changed by additional blockades. It is therefore wise for the BDEPT designer to subject their product to wide-angled scrutiny so weaknesses in their product can be uncovered at an early stage.

Aside from potential design flaws of BDEPT keeping it from the clinic, its momentum could be hampered by external forces. A major determinant of a medicine's quality concerns the types of institutions which contribute to its R&D. R&D of academic origin is sometimes reprimanded for its custom of turning out products that are unsatisfactory by industrial standards [178]. An awareness of this deficiency is particularly important, given that biotechnology companies, which act as industrial centres of innovation for therapies like BDEPT [179], are reliant on universities for the generation of many of their products [180]. It may be apt for academic BDEPT designers to liaise with industry sooner rather than later to permit synchronisation.

This review makes it clear that there is a complex line of questioning the BDEPT designer must pursue to create a workable product, yet this kind of therapy holds much promise to become a breakthrough mainstay of cancer treatment. The novelty of BDEPT makes it an especially lucrative avenue of R&D to explore, and its advent comes at a time of concern and precariousness in the pharmaceutical industry. A decline in R&D efficiency is evident, and it is speculated that it is becoming harder for novel medicines to reach the clinic, partly because of increasingly demanding regulatory authorities and the extremely high expectations of

healthcare standards set by existing treatments [181]. BDEPT is opportunely positioned to overstep these hurdles. There is a need in oncology for safe, targeted medicines, whereas for other indications such as hypertension and asthma, standards of care are already impressive and tough to improve on. This creates an entry point for novel oncological medicines, like BDEPT, which does not exist as obviously with medicines for other indications, for which the bar might be set much higher.

An indulgence in a field of drug discovery that has not yet begun to flourish may feel to the drug designer to be an overly risky endeavour. However, it is pertinent to recall that risk and prosperity in R&D go hand-in-hand [182]. Designers of BDEPT are potentially on the verge of forging a new, ground-breaking form of cancer therapy. How adeptly they will engage with this prospect will depend on their familiarity with the realities of R&D, and on their mastery of BDEPT design principles.

Conclusion

BDEPT possesses singular characteristics which differentiate it from other forms of gene therapy and directed enzyme prodrug therapy. These assets have placed it in the company of other promising treatments which aspire to control and cure malignant disease while minimising off-target damage. However, there are still obstacles which must be traversed before BDEPT can occupy a *de facto* position in the clinician's anticancer repertory.

BDEPT has experienced relatively little exposure to clinical testing thus far, but, to the extent that it has, it is notable that human subjects respond to treatment in a way that is sometimes inconsistent with preclinical findings. This disparity raises the question of how R&D practices should be strategized and enhanced to bridge the gap between clinical and preclinical observations.

Bacteria are undeniably adept at preferential accumulation in malignant tissues, yet clinical reports have pointed out that the targeting potential of these microbes may be restricted by dosage limitations that apply even to attenuated bacteria. This concern is reflected in the myriad studies which seek to reduce bacterial titres needed for tumour colonisation by, for example, the optimisation of enzyme kinetics. The co-option of synthetic biology to engineer superior tumour-targeting facilities into bacteria is also indicative of this concern.

This review provides a comprehensive guide to which researchers can refer as they continue to translate BDEPT into a clinically-viable medicine. Such a framework should prove especially useful at a time when BDEPT is still being tested clinically, amid abundant technological advancements which ought to work in its favour.

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Figure 1. Bacterial-directed enzyme prodrug therapy. The two-step procedure of BDEPT is illustrated, wherein bacteria expressing a prodrug-converting enzyme are administered intravenously to an oncology patient (step 1), followed by intravenous administration of a corresponding prodrug when the tumour is adequately colonised (step 2). The bacterial-mediated conversion of 5-fluorocytosine (a prodrug) to 5-fluorouracil (an API) is illustrated in step 2.

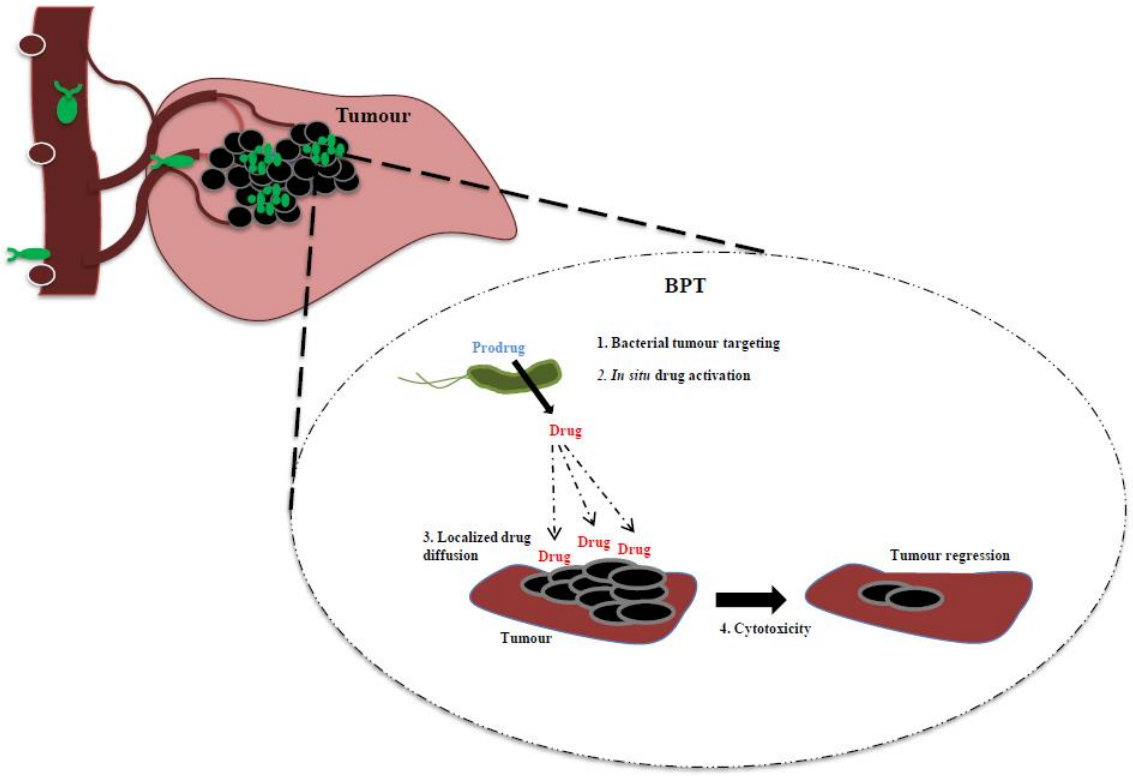
Figure 2. BDEPT Design Process Flow. An illustration of the order in which the de novo design of BDEPT should be approached. The process is divided into five distinct design principles. Curved arrows pointing from one design principle to another indicate a relationship in which an understanding of the design principle being pointed to is dependent on a familiarity with the design principle from which the arrow comes.

Figure 3. Enzymatic alignment strategies in BDEPT. An illustration of the various enzymatic conformations that have been investigated in BDEPT. The activation of hydrophilic prodrugs has been enhanced by coupling BDEPT enzymes to the surface or directing them to the periplasm of Gram-negative bacteria (e.g. *E. coli* BL21 and VNP20009, respectively).

Figure 4. Toxic metabolite production via BDEPT enzymes. Fludarabine is converted by PNP to 2-fluoroadenine (C5H5N5F), while CB1954 is converted by NTR to DNA cross-linking agents, all of which are potentially deleterious to bacteria via inhibition of DNA, RNA, and protein synthesis.

Figure 5. BDEPT enzymatic activity detection. Therapeutic enzymatic activity may be detected in situ, or ex vivo. Direct in vivo detection can be accomplished by activation of a light-emitting probe by bacterially-expressed enzymes at the site of the tumour, followed by MI to acquire an image of the probe. Indirect detection may be achieved by analysis of blood or urine samples for probes that have been activated by bacterial enzymes in the tumour and secreted into the circulation.

Figure 6. The application of synthetic biology to BDEPT. Synthetic biology rationalises BDEPT systems design by reducing its holistic structure to well-characterised, functional "parts" which, in concert with one another, are built into a modular "device" to carry out a predefined role. The device, when embedded in a chassis, forms a "system" which is fit to complete a task in accordance with its design. Within BDEPT, bacteria act as chassis for devices in the form of plasmids and chromosomes. These devices are equipped with various parts, e.g. secretion tags, which aim to coordinate the expression of a prodrug-converting enzyme.



Graphical abstract